# Carotid Body Function in Aged Rats: responses to Hypoxia, Ischemia, Dopamine and Adenosine

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## AT A GLANCE COMMENTARY

### Scientific Knowledge on the Subject

It is unknown what are the functional ventilatory consequences of carotid body morphological and molecular changes observed in aged animals, as well as the ventilatory effects of dopamine and adenosine in the old.

### What This Study Adds to the Field

Carotid body peripheral control of ventilation is not impaired in aging. The preservation of the inhibitory and excitatory effects on ventilation caused by exogenous dopamine and adenosine, respectively, should be taken in account in the therapeutic use in the old of these amines and their agonists and antagonists.

**Abstract**

**Rationale:** Carotid body (CB) is the main arterial chemoreceptor with a low threshold to hypoxia. CB activity is augmented by $A_2$-adenosine receptors stimulation and attenuated by $D_2$-dopamine receptors. The effect of aging on the ventilatory responses, mediated by the CB, to hypoxia, ischemia and to adenosine and dopamine administration is almost unknown.

**Objectives:** To investigate in old rats the ventilatory responses to ischemia and to adenosine, dopamine and their antagonists, as well as the effect of hypoxia on adenosine 3',5'-cyclic monophosphate (cAMP) accumulation in the CB.

**Methods:** *In vivo* experiments were performed on young and aged rats, anaesthetized with pentobarbitone and breathing spontaneously. CB ischemia was induced by bilateral common carotid occlusions. cAMP content was measured in CB incubated with different oxygen concentrations.

**Measurements and Main Results:** Hyperoxia caused a decrease in cAMP in the CB at all ages, but no differences were found between normoxia and hypoxia, as well as between young and old animals. Endogenous dopaminergic inhibitory tonus is slightly reduced, however both the decrease in ventilation caused by exogenous dopamine and the increase mediated by $A_{2A}$-adenosine receptors, is not impaired in aged animals. The bradycardia induced by adenosine is attenuated in old rats.

**Conclusions:** CB peripheral control of ventilation is preserved in ageing. Concerns to the clinical interest of adenosine to revert supraventricular tachycardia in aged people and to the use of dopamine in critical care situations were also advanced.

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cAMP, Adenosine $A_{2A}$ receptors

INTRODUCTION

At the periphery, mammalian carotid bodies (CBs) are the main chemoreceptor organs that control ventilation sensing $O_2$ ($PO_2$), $CO_2$ ($PCO_2$) and pH in arterial blood and initiating compensatory reflex responses via increased carotid sinus nerve (CSN) afferent activity, resulting in corrective changes in ventilation. CB glomus cells synaptically connected between them and with the afferent nerve terminals, depolarize in response to $PO_2$, $PCO_2$ and pH, releasing excitatory (acetylcholine, adenosine and ATP) or inhibitory (dopamine) transmitters (Iturriaga & Alcayaga, 2004).

It is known that ageing causes marked changes in the morphology of the CBs , reduces catecholamine release and CSN output in response to hypoxia in isolated CBs of rats (Conde *et al.*, 2006). In contrast, in healthy humans it has been suggested that peripheral chemoreceptor activity is not impaired by ageing (Smith *et al.*, 2001). Functional experiments *in vivo* models to deal with the gap between *in vitro* isolated preparations of the CBs and humans are missing.

Occlusions of the common carotid artery during short periods (s) have been used as a functional model of accessing peripheral chemoreceptor responses (Alcayaga *et al.*, 1986). The effects of ageing on the cardiorespiratory reflexes induced by carotid ischemia have never been studied.

Dopamine has inhibitory effects on ventilation at rest (Zapata & Zuazo, 1980) and in the presence of hypoxic exposure (Nishino & Lahiri, 1981) mediated by $D_2$-receptors localized at the CB (Gonzalez *et al.*, 1994). No data are available concerning the effects of ageing on $D_2$-receptors at the CB.

$A_2$-adenosine receptors at the CB are involved in the stimulation of breathing in basal conditions (McQueen & Ribeiro, 1986;Monteiro & Ribeiro, 1987) and in response to hypoxia (Conde & Monteiro, 2004).

In the present work, CB function was assessed measuring the ventilatory responses induced by acute CB ischemia and exogenous administrations of adenosine and dopamine and their receptor antagonists. Changes in cAMP content, in response to oxygen concentrations in aged rats, were also investigated as a cellular indicator of CB function. cAMP has been postulated to modulate the response of the CB to hypoxia, and is the common pathway of the activation of both dopamine and adenosine receptors at the CB (Batuca *et al.*, 2003). The effects of ageing on cAMP production at the CB have never been addressed. An additional reason to test this hypothesis is that age-related alterations in the adenylate cyclase/cAMP system have been documented in other structures. For example, impairment of the activity of the catalytic subunit of adenylate cyclase was observed in rats (Kilts *et al.*, 2002), and in the heart of > 60 year old humans (Brodde *et al.*, 1995). However, on the contrary, it has

been described that the maximal ability of forskolin to increase cAMP in the adrenal medulla and liver is enhanced in 24 month old rats (Tumer *et al.*, 1996).

The results obtained in the present work will also contribute to investigate whether changes in the magnitude of the cardiorespiratory responses induced by dopamine and adenosine administration for clinical purposes (shock and supraventricular tachycardia, respectively) would be expected in elder people.

## METHODS

### Animals and House Conditions

Experiments were performed on male Wistar rats aged 3, 12 and 24 months. The animals were housed in the vivarium of the university, in an air-conditioned room at 21±1°C, 55±10% humity, with a 12:12 h light/dark cycle (with lights on at 08:00 and off at 20:00), and with food and water available *ad libitum*. All surgical procedures and experimental protocols were handled in accordance with the EU guidelines for use of experimental animals (86/609/EEC), with care to minimize the number of animals used and their suffering. The investigators own FELASA level C certification and euthanasia of the rats was achieved by i.v. injection of a lethal dose of pentobarbital sodium (180 mg.$Kg^{-1}$ iv).

### *In vivo* Experiments

A detailed description of these methods was previously published (Monteiro & Ribeiro, 1987;Monteiro & Ribeiro, 1989) and can be found in the online supplement. Briefly, respiratory frequency ($f_R$), tidal volume ($V_T$), blood pressure (BP) and heart rate (HR) were continuously recorded in anesthetized and vagotomized rats breathing spontaneously and submitted to bilateral occlusions (5-15 s) of the common carotid artery, or to drugs administered into de common carotid artery or femoral vein. Control experiments to distinguish central and peripheral mediated effects were performed in animals after bilateral denervation of the CBs by cutting the CSN.

### Experimental protocols with cAMP

Immediately after surgical removal from the carotid bifurcation of anaesthetized rats the CBs were submitted to similar experimental conditions published elsewhere (Batuca *et al.*, 2003) and described in the online supplement. Essentially, CBs were incubated in solutions, containing 500µM of isobutylmethylxanthine (IBMX) equilibrated with 95 %$O_2$/5 %$CO_2$ (hyperoxia), 20 %$O_2$/5 %$CO_2$/75 %$N_2$ (normoxia), 10 %$O_2$/5 %$CO_2$/85 %$N_2$ (mild hypoxia) or 5 %$O_2$/5 %$CO_2$/90 %$N_2$ (moderately intense hypoxia), during 30 min. cAMP was assayed using an EIA commercial kit (GE Healthcare Bio-Sciences AB, Sweden).

**Statistics**

In *in vivo* experiments, each animal served as its own control. To facilitate comparisons between young and old animals, cardiorespiratory data are expressed as percent change. Data are expressed as mean ± S.E.M. and statistical significance was evaluated by using the Student's paired and unpaired t-test for *in vivo* experiments and one-way analysis of variance for *in vitro* experiments, with p values < 0.05 taken as significant. Models for analysis were developed using GraphPad Prism software (Version 4.03).

**Drugs**

All drugs were prepared on the day of each experiment. Doses of all drugs were calculated on the basis of salt weight. Dopamine, adenosine and domperidone were prepared in saline (0.9% NaCl). Stock solutions (5mM) of SCH 58261 was prepared in dimethylsulfoxide (DMSO) and stored at –20ºC until use. Stock solution was further diluted with saline prior to each experiment. The highest concentration of the vehicle in venous perfusion was 0.4 mM or 0.01%.

Adenosine (Adenocor) was obtained from Sanofi-synthelabo (Portugal).

Dimethyl-sulfoxide (DMSO) was obtained from J.T.Baker (Holland).

Domperidone was obtained from Sigma-RBI Chemical (Potugal/USA).

Dopamine (Medopa) was obtained from Medinfar (Portugal).

Heparine sodium was obtained from B. Braun Medical (Portugal).

Isobutylmethylxanthine (IBMX) was obtained from Sigma-Aldrich (Portugal)

SCH 58261 (7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazole-[4,3-e]-1,2,4-triazolo[1,5-c] pyrimidine) was obtained from Sigma-RBI Chemical (Potugal/USA).

Sodium pentobarbitone (Eutasil) was obtained from Sanofi-Veterinária (Miraflores, Algés,Portugal).

## RESULTS

### Age-Related Evolution of the Body Weight

Figure 1 shows the age-related increase in body size in rats between 3 and 24 months. Statistical significant differences were found between body weight in 3 months old animals (431.0±7.4 g, n=30) and older ages. No differences were observed between weight in 12 (618.1±16.9g) and 24 (641.0±11.3 g) months old animals.

### Effects of Ageing in Resting Ventilatory and Cardiovascular Parameters

The effects of ageing on resting cardioventilatory parameters in anaesthetised rats are illustrated in Figure 2. The basal values of $f_R$ were maintained constant throughout ageing (49.8±1.2, 52.26±1.5 and 46.6±1.5 breaths.min$^{-1}$, respectively in 3, 12 and 24 months old rats). Whereas significant decreases ($p<0.01$) in $V_T$ (8.8±0.3, 6.0±0.3 and 7.0±0.4 mL.Kg$^{-1}$, respectively in 3, 12 and 24 months old rats) and $V_E$ (442.5±24.2, 316.4±12.4 and 322.9±18.8 ml.min$^{-1}$.Kg$^{-1}$, respectively in 3, 12 and 24 months old rats), decreased significantly ($p<0.01$) after were evident in 12 months and older animals (Figure 2A, 2B and 2C).

Basal values of HR and BP at 12 months old were 322.2±9.2 beats.min$^{-1}$ and 92.6±5.4 mmHg, respectively. These values were lower than those observed in 3 months old animals but not statistically different than those recorded in older animals (Figure 2D and 2E)

### Effect of Ageing on Ventilatory Responses Induced by Common Carotid Occlusions

Bilateral CCO during 5, 10 and 15 s induced a time-dependent excitatory effect on ventilation (Figure 3) that is totally abolished by carotid sinus nerve section. This excitatory effect on ventilation caused by carotid ischemic stimuli was preserved in old rats (Figure 3). The transient increases in BP observed in young and old animals during CCO (Figure 3A and 3B), were similar in both groups. No apparent changes in HR were detected during CCO in the 3 groups of animals.

### Cardioventilatory Responses to Dopamine

Dose-response curves for the effects of i.c. bolus injections of dopamine (3-100 nmol) on cardioventilatory parameters in anestethized and vagotomized rats are showed in Figure 4. The inhibitor effect of dopamine on ventilation, and its slight increases in HR and BP were dose-dependent and of the same magnitude in both young adults and aged rats. As previously shown by others (Zapata & Zuazo, 1980), bilateral section of the CSN completely abolished the inhibitory effect caused by dopamine on $f_R$, $V_T$ and $V_E$ but did not change the increase in BP (not shown). The effect of dopamine on cardioventilatory parameters was also tested in the presence of

domperidone, a dopamine $D_2$–receptor selective antagonist that does not cross the blood-brain barrier. Domperidone (0.01-0.5 mg.$Kg^{-1}$, $min^{-1}$/2.35-137.5 μmol. $Kg^{-1}$, $min^{-1}$; i.v. infusion) almost totally abolished the depression on $V_E$ induced by dopamine (Figure 5). The antagonism is dose-dependent, and of the same magnitude in 3, 12 and 24 month old rats (Figure 5A, 5B and 5C). In contrast, domperidone (0.01-0.5 mg.$Kg^{-1}$.$min^{-1}$, i.v.) did not modify the effects of dopamine on BP and HR either in young or aged animals (Figure 5D and 5E). The effect of domperidone infusion (0.01-0.1 mg.$Kg^{-1}$.$min^{-1}$) by itself in the absence of exogenous dopamine, on $V_E$ in 3 and 24 months old rats is illustrated in Figure 6. Domperidone alone caused statistically significant increases in basal $V_E$ in both young and aged animals in a dose-dependent manner. Although the excitatory effect of this $D_2$-dopamine antagonist on ventilation is significantly ($p<0.01$) attenuated in aged animals (Figure 6A). The maximal excitatory effect (66.8±8.1 breaths.$Kg^{-1}$.$min^{-1}$) was achieved with 0.1 mg.$Kg^{-1}$. $min^{-1}$ in young animals. The higher dose (0.5 mg.$Kg^{-1}$.$min^{-1}$) caused a less pronounced (44.3±12.9 %) effect on ventilation. Domperidone (0.01 and 0.1 mg.$Kg^{-1}$.$min^{-1}$) by itself did not cause apparent modifications in both HR and BP.

**Cardioventilatory Responses to Adenosine**

The results obtained in the experiments performed to investigate whether the excitatory effect of adenosine on ventilation was modified by age are shown in Figure 7. Dose-response curves for the effects of i.c. bolus injections of adenosine (3-100nmol) on $f_R$, $V_T$, $V_E$, HR and BP were obtained in 3 months and 24 months old rats. As expected, adenosine by itself caused excitatory effects on $V_E$ due to increases in both $f_R$ and $V_T$ (Figure 7A, 7B and 7C). Adenosine 100 nmol, increased $V_E$ by 60.9±2.9 and 55.1±3.7 %, respectively in 3 and 24 months old rats. The responses in $f_R$, $V_T$ and $V_E$ to exogenously administered adenosine were remarkably similar in 3 and in 24 months old rats (Figure 7A, 7B and 7C). As previously shown by others (Monteiro & Ribeiro, 1987), respiratory effects of adenosine were abolished by CSN section (not shown). An immediate decrease in the HR and BP that remained in animals after bilateral section of the CSN was also induced by exogenous adenosine. The hypotensive and bradycardic effect of adenosine (100nmol) were clearly attenuated in 24 month old rats (Figure 7D and 7E).

In a group of experiments, i.c. bolus injections of adenosine were performed during i.v. infusion of an $A_{2A}$-adenosine receptor antagonist, SCH 58261. The effect of adenosine 100 nmol on $V_E$ was fully abolished by SCH 58261, 20 ng.$Kg^{-1}$.$min^{-1}$, in both young and old animals (Figure 8A and 8B). Figure 8 also depicts that adenosine $A_{2A}$-receptors blockade (SCH 58261; 2-20 ng.$Kg^{-1}$.$min^{-1}$ i.v.) did not prevent the decrease in HR and BP evoked

by adenosine in both young and old animals. In contrast, a small potentiating of the bradycardic effect of adenosine by SCH 58261 was observed in old animals (Figure 8D).

SCH58261 alone in the dose of 2 $ng.Kg^{-1}.min^{-1}$ i.v., significantly ($p>0.01$) increased $V_E$ (18.5±0.5 and 16.4±0.8 %, respectively in 3 and 24 months old rats), whereas the dose of 20 $ng.Kg^{-1}.min^{-1}$ i.v. did not cause by itself, appreciable changes in $V_E$ in both 3 and 24 months old rats (Figure 9).

Administration of the adenosine $A_{2A}$-receptor antagonist SCH 58261 alone had no effect on HR or BP (Figure 9).

**Effects of Age on cAMP Levels in CBs in Response to Different Oxygen Concentrations**

The cAMP levels in CBs isolated from 3, 12, 18 and 24 months old rats, incubated in normoxic solutions (20%O2) are shown in Figure 10A. No differences were found between cAMP levels in CBs of young and old rats expressed by CB or corrected by the CB weight at different ages (Figure 10A). The weights of the CBs (μg) slightly changes throughout aging and were: 49 ± 7 (3 months), 46 ± 8 (12 months), 75 ± 12 (18 months) and 70 ± 10 (24 months).

Figure 10B shows the effect of aging on cAMP production induced by different oxygen concentrations applied to the CBs. $PO_2$ of around 677 mmHg (incubating solutions equilibrated with 95 $\%O_2$ / 5% $CO_2$) yields cAMP levels significantly lower than those found when the incubating $PO_2$ is close to physiological or normoxic (≈ 142 mmHg; incubating solutions equilibrated with 20 $\%O_2$ / 5 $\%CO_2$). In the CB, the normoxic level of cAMP was maintained at lower $PO_2$ of ≈ 71 (10%O2) and 35 mmHg (5% O2). This pattern and the amount of cAMP production by the carotid bodies did not change with aging.

DISCUSSION

This functional approach of carotid body chemoreceptors activity that included ventilatory responses to ischemia, pharmacological manipulation of two important carotid body neuromediators and intracellular cAMP accumulation induced by changes in $O_2$, showed that peripheral control of ventilation is not apparently impaired in ageing.

The ageing process is characterized by a decline in several physiological functions, with the result of a reduction in the ability to maintain homeostasis (Troen, 2003). However, there is an increasingly body of evidence that several physiological functions are well preserved in ageing. Namely, several studies in humans have examined the age-related changes in ventilatory response to hypoxia and found a maintained ventilatory response

throughout aging, suggesting no alteration in peripheral quimoreception in the old humans (Pokorski *et al.*, 2004;Smith *et al.*, 2001;Vovk *et al.*, 2004).

The present work contributes indirectly to know the effect of ageing in the oxygen-sensing mechanisms, and was focused in the peripheral control of ventilation mediated by the CB.

It was previously described that respiratory frequency decreases with increasing size of the animals and declined in a linear fashion with increasing age in rat (Soulage *et al.*, 2004). Healthy elderly subjects at rest breathe with a $V_E$ identical to that of younger subjects, but with smaller $V_T$ and higher $f_R$ (Janssens *et al.*, 1999). In the experimental conditions of our model, a significant increase in rat weight was found after 12 months old, but the basal values of $f_R$ were maintained constant throughout ageing. Since the animals were vagotomized and under the influence of sodium pentobarbitone anaesthesia showed lower values of $f_R$ (46.6±1.5 breaths.min$^{-1}$) which could explain the absence of bradypnea found by others in old rats where basal values of $f_R$ were 104.7±3.4 breaths.min$^{-1}$. The absence of changes in $f_R$ when the input of the major pulmonary stretch receptors is abolished by vagotomy and CNS activity depressed by the barbiturate, suggest that changes in $f_R$ in the elderly could not be attributed to an impairment of the CB peripheral drive.

Wistar rats exhibited, in the present conditions, a diminution of tidal volume with ageing. Although some authors did not find tidal volume to decrease with advancing age (Soulage *et al.*, 2004), an age-related decrease were commonly observed in rats (Nagase *et al.*, 1994) and humans (Janssens *et al.*, 1999) with advancing age, and have been linked to alterations of the mechanical properties of lung and thorax (Chan & Welsh, 1998).

The cardiovascular effects associated with ageing are: a striking attenuation in the cardiac frequency in humans (Kronenberg & Drage, 1973); no significant effect of age on resting HR of rats (Gordon, 2008) and increase in blood pressure in both rats (Di *et al.*, 2009) and humans (Fleg *et al.*, 1995). In the present work mean arterial blood pressure was lower in 24 months old rats than in young adults. This finding could be attributed to the predominant effect of anaesthetic mediated central cardiovascular depression (Schwenke & Cragg, 2004) over the arterial dysfunction in ageing. It is known that the risk of cardiovascular depression induced by barbiturates is higher in the elderly (Schwenke & Cragg, 2004) and compatible with the lower values for BP found in the present work, despite lower doses of anaesthetic were needed to abolish sensitivity in old animals.

There is a consensual body of evidence about the changes in the morphology of the CB throughout ageing: increase in extracellular matrix, reduction in number and volume of type I cells, and in the volume and density of mitochondria compared with young CB (Conde *et al.*, 2006;Pokorski *et al.*, 2004;Hurst *et al.*, 1985). These

morphological findings do not apparently agree with the absence of functional impairment, but the increases in the number of type II stem cells could contribute to the maintenance of CB function (Hurst *et al.*, 1985;Porzionato *et al.*, 2005). The present work supports previous findings in humans - absence of alterations in the peripheral control of ventilatory responses to hypoxia (Pokorski *et al.*, 2004;Vovk *et al.*, 2004), providing evidence that structural and neurochemical changes caused by ageing at the carotid body are further compensated. The finding that changes in cAMP content at the CB in response to $O_2$ concentrations did not change with ageing, suggests that the compensatory mechanism could occur within the CB as a whole. These results contrast with the reduction in carotid sinus nerve discharges in response to hypoxia observed in old rats (Conde *et al.*, 2006). It is known that the glossopharyngeal nerve has, in addition to carotid sinus nerve afferent fibres, a parallel autonomic parasympathetic efferent pathway, sensitive to hypoxia that is the source of CB inhibition (Campanucci *et al.*, 2006). We can speculate that the reduction in CSN discharges found in old animals) (Conde *et al.*, 2006) could be mainly due to an impairment of the efferent fibre activity without changes in chemosensory excitatory afferent pathway. Similarly, it was shown in humans that ageing impairs the autonomic responses to pain but does not modify nociceptive perception (Hajduczok *et al.*, 1991).

Hyperventilation induced by bilateral brief (<15s) occlusions of the common carotid arteries are abolished by carotid sinus nerve section (Monteiro & Ribeiro, 1989), constituting an alternative model to hypoxia in order to study CB chemosensory activation *in vivo*. In the present work the cardiorespiratory reflexes induced by carotid ischemia were similar in young and old rats. The effects of ageing on the ventilatory reflexes induced by carotid ischemia were here shown for the first time. Carotid sinus baroreflex function throughout ageing has never been studied in this model but maintenance of carotid baroreflex function in advanced aged rats was previously described in anaesthetized Fisher rats (Wei *et al.*, 1986). In humans, there is no difference in carotid body baroreflexes throughout ageing (Fiocchi *et al.*, 1985;Shi *et al.*, 1996).

Other aspect to consider in our discussion relates to the age-dependent variations in cAMP levels and responses to hypoxia. Ageing did not cause appreciable changes in cAMP levels at the CB. The normoxic cAMP levels are not different at the four ages studied, and the pattern of cAMP production in response to different $O_2$ concentrations remained almost unchanged in old animals. Expressed by unit weight, the levels of cAMP found for the rat CB in the present study are nearly identical to the values reported in the rabbit CB (Chen *et al.*, 1997;Perez-Garcia *et al.*, 1990). At all ages, cAMP levels were maximal in normoxic conditions. In comparison to normoxia, hyperoxia caused a decrease in cAMP in the CB at all ages. Adenosine effects via $A_2$ receptors, which are known to be expressed in the CB (Gauda, 2002), can represent one of the mechanisms maintaining

high cAMP levels in hypoxia. It is worth noting that the release of adenosine in the CB is maximal at mild levels of hypoxia (10 $\%O_2$), decreasing with higher intensities of hypoxic stimulation and that the most intense hypoxia used in this study does not increase the release of adenosine in the SCG or CA (Conde & Monteiro, 2004). Of course adenosine is not the only modulator of cAMP levels in the CB, as it is well known that hypoxia also increases the release of many other neurotransmitters, particularly dopamine (Vicario *et al.*, 2000). It is also known that the CB expresses high levels of $D_2$ dopamine receptors (Gauda, 2002) which are negatively coupled to adenylate cyclase (Kebabian & Calne, 1979). In fact, it would appear that the much higher rate of dopamine release in the rat *vs.* the rabbit CB in hypoxia (Vicario *et al.*, 2000) can explain the inability of hypoxia to increase the cAMP levels in the rat CB above those found in normoxia, as has previously been observed in the rabbit (Cachero *et al.*, 1996). It should be mentioned that Mir et al. (Mir *et al.*, 1983), in their pioneer study, also found that 5% $O_2$ administered *in vivo* did not significantly modify cAMP levels in the rat CB.

The absence of significant changes in cAMP production in old animals is not incompatible with individual changes in adenylate cyclase activity, and/or metabotropic receptors but means that if individual changes occurred, the overall response is maintained.

Effects of aging in cAMP accumulation in other preparations shows impairment of the activity of the catalytic subunit of adenylate cyclase in rats (Kilts *et al.*, 2002), and in the heart of >60 year old humans (Brodde *et al.*, 1995). However, on the contrary, it has been described that the maximal ability of forskolin to increase cAMP in the adrenal medulla and liver is enhanced in 24 month old rats (Tumer *et al.*, 1996).

If a reduction in both $A_2$-adenosine (positively coupled to adenylate cyclase) and $D_2$-dopamine (negatively coupled to adenylate cyclase) receptor-mediated responses coexist at the CB, then compensatory effects in cAMP production can take place. Actually, the results obtained in the present work with exogenous adenosine and dopamine as well as manipulating their endogenous effects through antagonists showed that neither adenosine or dopamine receptors are significantly impaired in old animals. These effects of dopamine and adenosine were mediated through carotid body chemoreceptors, since these actions disappeared after bilateral section of the carotid sinus nerves.

An additional interest to test the effects of dopamine and adenosine in the present model is their specific therapeutic indications in clinical practice: shock and supraventricular tachycardia, respectively. These clinical situations are associated with heart failure, more prevalent in the elderly, and the cardiorespiratory effects of exogenous dopamine and adenosine in aged humans or animals have never been investigated.

Dopamine has been shown to impair the ventilatory drive in response to hypoxemia and depresses minute ventilation and oxygen saturation in heart failure patients even when they are breathing room air (van de *et al.*, 1998). The present work provides evidence that these undesirable effect should be of the same magnitude in aged subjects.

Domperidone, a $D_2$-selective antagonist that do no cross the blood brain barrier (Baudry *et al.*, 1979) administered alone increased basal $V_E$ as described in previous work (Gamboa *et al.*, 2003;Lahiri *et al.*, 1984) and we found statistically significant differences between 3 and 24 months old rats. This means that the inhibitory basal tonus of dopamine at the CB activity is less pronounced in old rats, probably resulting from a diminution of the receptor number throughout ageing. This is not an unexpected finding considering that it has been previously found that the density of striatal $D_2$-receptors is significantly reduced in aged rats from 30 to 80% depending on the study (Han *et al.*, 1989;Marshall & Joyce, 1988;Petkov *et al.*, 1988;Popoli *et al.*, 1998). Anyway, at the CB this reduction is not enough to reduce the effect of exogenous dopamine which has the same magnitude in old rats and controls.

The BP response triggered by dopamine, a short-lived rise followed to a return to baseline, occurred independent of the carotid sinus nerve afferentation and blockade of dopamine $D_2$-receptors. This result warrants that the prompt hypertensive response evoked by dopamine challenge apparently occurs beyond the baroreceptor afferentation and may depend on $D_1$-dopamine receptors at the heart and/or adrenoceptors.

Adenosine, despite its dyspnoeic effect mediated by chemoreceptor activation (Burki *et al.*, 2005), is a clinical useful tool to treat supraventricular tachycardia (Biaggioni *et al.*, 1987;Riccardi *et al.*, 2008), a common cardiac rhythm disturbance more frequently observed in the elderly (Medi *et al.*, 2009). However, studies about this effect on aged subjects are missing. Although the present work was not focused in the crono/dromo and batmotropic effects of adenosine, an interesting finding is the less bradycardic effect observed during its exogenous application in aged animals, suggesting that the efficacy of adenosine to revert supraventricular tachycardia could be attenuated in the elderly. This could be attributed to a reduction in the density of adenosine receptors in the aged heart and may also involve an age-related reduction in the intrinsic ability of nodal tissue to respond to adenosine receptor activation (Hinschen *et al.*, 2001). Some authors find an absence of changes in $A_1$-receptors density and G alpha protein levels but an adenosine $A_1$-receptor function in rat ventricles to decrease with age and this was related to a reduction in the coupling between adenosine $A_1$-receptor and their G proteins (Cai *et al.*, 1997).

The excitatory effects of adenosine found in old rats suggest that the $A_2$ -adenosine receptors at the carotid body are well preserved and that the chest discomfort caused by its exogenous administration will remain in the elderly. The excitatory effects obtained with low doses of SCH 58261 by itself, an adenosine $A_{2A}$-antagonist that crosses the blood brain barrier (El *et al.*, 2000), are in agreement with the presence of $A_{2A}$ receptors with inhibitory effects on the control of breathing (Mayer *et al.*, 2006) and that central inhibitory control is apparently more relevant in basal conditions than the excitatory effects peripherally mediated. Therefore, low doses of $A_{2A}$ antagonists that cross BBB like SCH 58261 could be useful to treat central mediated ventilatory depressions without major actions in the cardiovascular system because the doses that antagonize the ventilatory effects did not modify BP and HR.

In summary, we found that carotid body peripheral control of ventilation is not impaired with ageing. At the carotid body, endogenous dopaminergic inhibitory tonus is slightly reduced, however the decrease in ventilation caused by exogenous dopamine is preserved in aged animals, and should be taken in account in its use in critical care situations. The excitatory effect of exogenous adenosine mediated by $A_{2A}$ receptors at the carotid body chemosensors was not modified in ageing. In contrast, the bradycardic effect of exogenous adenosine mediated by $A_1$ receptors is attenuated in old animals. These findings reduce the clinical interest of adenosine to revert supraventricular tachycardia in aged people. The maintained carotid body function throughout ageing confirms this organ as a valuable model of successful ageing.

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References

Alcayaga J, Iturriaga R, & Zapata P (1986). Carotid body chemoreceptor excitation produced by carotid occlusion. *Acta Physiol Pharmacol Latinoam* **36**, 199-215.

Batuca JR, Monteiro TC, & Monteiro EC (2003). Contribution of dopamine D2 receptors for the cAMP levels at the carotid body. *Adv Exp Med Biol* **536**, 367-373.

Baudry M, Martres MP, & Schwartz JC (1979). 3H-Domperidone: a selective ligand for dopamine receptors. *Naunyn Schmiedebergs Arch Pharmacol* **308**, 231-237.

Biaggioni I, Olafsson B, Robertson RM, Hollister AS, & Robertson D (1987). Cardiovascular and respiratory effects of adenosine in conscious man. Evidence for chemoreceptor activation. *Circ Res* **61**, 779-786.

Brodde OE, Zerkowski HR, Schranz D, Broede-Sitz A, Michel-Reher M, Schafer-Beisenbusch E, Piotrowski JA, & Oelert H (1995). Age-dependent changes in the beta-adrenoceptor-G-protein(s)-adenylyl cyclase system in human right atrium. *J Cardiovasc Pharmacol* **26**, 20-26.

Burki NK, Dale WJ, & Lee LY (2005). Intravenous adenosine and dyspnea in humans. *J Appl Physiol* **98**, 180-185.

Cachero TG, Rigual R, Rocher A, & Gonzalez C (1996). Cholera and pertussis toxins reveal multiple regulation of cAMP levels in the rabbit carotid body. *Eur J Neurosci* **8**, 2320-2327.

Cai G, Wang HY, Gao E, Horwitz J, Snyder DL, Pelleg A, Roberts J, & Friedman E (1997). Reduced adenosine A1 receptor and G alpha protein coupling in rat ventricular myocardium during aging. *Circ Res* **81**, 1065-1071.

Campanucci VA, Zhang M, Vollmer C, & Nurse CA (2006). Expression of multiple P2X receptors by glossopharyngeal neurons projecting to rat carotid body O2-chemoreceptors: role in nitric oxide-mediated efferent inhibition. *J Neurosci* **26**, 9482-9493.

Chan ED & Welsh CH (1998). Geriatric respiratory medicine. *Chest* **114**, 1704-1733.

Chen J, Dinger B, & Fidone SJ (1997). cAMP production in rabbit carotid body: role of adenosine. *J Appl Physiol* **82**, 1771-1775.

Conde SV & Monteiro EC (2004). Hypoxia induces adenosine release from the rat carotid body. *J Neurochem* **89**, 1148-1156.

Conde SV, Obeso A, Rigual R, Monteiro EC, & Gonzalez C (2006). Function of the rat carotid body chemoreceptors in ageing. *J Neurochem* **99**, 711-723.

Di NF, Burattini R, Cogo CE, Faelli E, & Ruggeri P (2009). Age-related analysis of insulin resistance, body weight and arterial pressure in the Zucker fatty rat. *Exp Physiol* **94**, 162-168.

El YM, Ledent C, Parmentier M, Costentin J, & Vaugeois JM (2000). The anxiogenic-like effect of caffeine in two experimental procedures measuring anxiety in the mouse is not shared by selective A(2A) adenosine receptor antagonists. *Psychopharmacology (Berl)* **148**, 153-163.

Fiocchi R, Fagard R, Vanhees L, Grauwels R, & Amery A (1985). Carotid baroreflex sensitivity and physical fitness in cycling tourists. *Eur J Appl Physiol Occup Physiol* **54**, 461-465.

Fleg JL, O'Connor F, Gerstenblith G, Becker LC, Clulow J, Schulman SP, & Lakatta EG (1995). Impact of age on the cardiovascular response to dynamic upright exercise in healthy men and women. *J Appl Physiol* **78**, 890-900.

Gamboa J, Macarlupu JL, Rivera-Chira M, Monge C, & Leon-Velarde F (2003). Effect of domperidone on ventilation and polycythemia after 5 weeks of chronic hypoxia in rats. *Respir Physiol Neurobiol* **135**, 1-8.

Gauda EB (2002). Gene expression in peripheral arterial chemoreceptors. *Microsc Res Tech* **59**, 153-167.

Gonzalez C, Almaraz L, Obeso A, & Rigual R (1994). Carotid body chemoreceptors: from natural stimuli to sensory discharges. *Physiol Rev* **74**, 829-898.

Gordon CJ (2008). Cardiac and thermal homeostasis in the aging Brown Norway rat. *J Gerontol A Biol Sci Med Sci* **63**, 1307-1313.

Hajduczok G, Chapleau MW, Johnson SL, & Abboud FM (1991). Increase in sympathetic activity with age. I. Role of impairment of arterial baroreflexes. *Am J Physiol* **260**, H1113-H1120.

Han Z, Kuyatt BL, Kochman KA, DeSouza EB, & Roth GS (1989). Effect of aging on concentrations of D2-receptor-containing neurons in the rat striatum. *Brain Res* **498**, 299-307.

Hinschen AK, Rose'Meyer RB, & Headrick JP (2001). Age-related changes in adenosine-mediated relaxation of coronary and aortic smooth muscle. *Am J Physiol Heart Circ Physiol* **280**, H2380-H2389.

Hurst G, Heath D, & Smith P (1985). Histological changes associated with ageing of the human carotid body. *J Pathol* **147**, 181-187.

Iturriaga R & Alcayaga J (2004). Neurotransmission in the carotid body: transmitters and modulators between glomus cells and petrosal ganglion nerve terminals. *Brain Res Brain Res Rev* **47**, 46-53.

Janssens JP, Pache JC, & Nicod LP (1999). Physiological changes in respiratory function associated with ageing. *Eur Respir J* **13**, 197-205.

Kebabian JW & Calne DB (1979). Multiple receptors for dopamine. *Nature* **277**, 93-96.

Kilts JD, Akazawa T, Richardson MD, & Kwatra MM (2002). Age increases cardiac Galpha(i2) expression, resulting in enhanced coupling to G protein-coupled receptors. *J Biol Chem* **277**, 31257-31262.

Kronenberg RS & Drage CW (1973). Attenuation of the ventilatory and heart rate responses to hypoxia and hypercapnia with aging in normal men. *J Clin Invest* **52**, 1812-1819.

Lahiri S, Smatresk N, Pokorski M, Barnard P, Mokashi A, & McGregor KH (1984). Dopaminergic efferent inhibition of carotid body chemoreceptors in chronically hypoxic cats. *Am J Physiol* **247**, R24-R28.

Marshall JF & Joyce JN (1988). Basal ganglia dopamine receptor autoradiography and age-related movement disorders. *Ann N Y Acad Sci* **515**, 215-225.

Mayer CA, Haxhiu MA, Martin RJ, & Wilson CG (2006). Adenosine A2A receptors mediate GABAergic inhibition of respiration in immature rats. *J Appl Physiol* **100**, 91-97.

McQueen DS & Ribeiro JA (1986). Pharmacological characterization of the receptor involved in chemoexcitation induced by adenosine. *Br J Pharmacol* **88**, 615-620.

Medi C, Kalman JM, & Freedman SB (2009). Supraventricular tachycardia. *Med J Aust* **190**, 255-260.

Mir AK, Pallot DJ, & Nahorski SR (1983). Biogenic amine-stimulated cyclic adenosine-3',5'-monophosphate formation in the rat carotid body. *J Neurochem* **41**, 663-669.

Monteiro EC & Ribeiro JA (1989). Inhibition by 1,3-dipropyl-8(p-sulfophenyl)xanthine of the respiratory stimulation induced by common carotid occlusion in rats. *Life Sci* **45**, 939-945.

Monteiro EC & Ribeiro JA (1987). Ventilatory effects of adenosine mediated by carotid body chemoreceptors in the rat. *Naunyn Schmiedebergs Arch Pharmacol* **335**, 143-148.

Nagase T, Fukuchi Y, Teramoto S, Matsuse T, & Orimo H (1994). Mechanical interdependence in relation to age: effects of lung volume on airway resistance in rats. *J Appl Physiol* **77**, 1172-1177.

Nishino T & Lahiri S (1981). Effects of dopamine on chemoreflexes in breathing. *J Appl Physiol* **50**, 892-897.

Perez-Garcia MT, Almaraz L, & Gonzalez C (1990). Effects of different types of stimulation on cyclic AMP content in the rabbit carotid body: functional significance. *J Neurochem* **55**, 1287-1293.

Petkov VD, Petkov VV, & Stancheva SL (1988). Age-related changes in brain neurotransmission. *Gerontology* **34**, 14-21.

Pokorski M, Walski M, Dymecka A, & Marczak M (2004). The aging carotid body. *J Physiol Pharmacol* **55 Suppl 3**, 107-113.

Popoli P, Betto P, Rimondini R, Reggio R, Pezzola A, Ricciarello G, Fuxe K, & Ferre S (1998). Age-related alteration of the adenosine/dopamine balance in the rat striatum. *Brain Res* **795**, 297-300.

Porzionato A, Macchi V, Guidolin D, Parenti A, Ferrara SD, & De CR (2005). Histopathology of carotid body in heroin addiction. Possible chemosensitive impairment. *Histopathology* **46**, 296-306.

Riccardi A, Arboscello E, Ghinatti M, Minuto P, & Lerza R (2008). Adenosine in the treatment of supraventricular tachycardia: 5 years of experience (2002-2006). *Am J Emerg Med* **26**, 879-882.

Schwenke DO & Cragg PA (2004). Comparison of the depressive effects of four anesthetic regimens on ventilatory and cardiovascular variables in the guinea pig. *Comp Med* **54**, 77-85.

Shi X, Gallagher KM, Welch-O'Connor RM, & Foresman BH (1996). Arterial and cardiopulmonary baroreflexes in 60- to 69- vs. 18- to 36-yr-old humans. *J Appl Physiol* **80**, 1903-1910.

Smith WD, Poulin MJ, Paterson DH, & Cunningham DA (2001). Dynamic ventilatory response to acute isocapnic hypoxia in septuagenarians. *Exp Physiol* **86**, 117-126.

Soulage C, Pequignot JM, & Perrin D (2004). Breathing pattern and hypoxic sensitivity during ageing in a new model of obesity-resistant rat. *Respir Physiol Neurobiol* **144**, 45-57.

Troen BR (2003). The biology of aging. *Mt Sinai J Med* **70**, 3-22.

Tumer N, Sego RL, & Scarpace PJ (1996). Atypical pattern of adenylyl cyclase activity in the adrenal medulla with age. *Exp Gerontol* **31**, 571-576.

van de BP, Oren R, & Somers VK (1998). Dopamine depresses minute ventilation in patients with heart failure. *Circulation* **98**, 126-131.

Vicario I, Rigual R, Obeso A, & Gonzalez C (2000). Characterization of the synthesis and release of catecholamine in the rat carotid body in vitro. *Am J Physiol Cell Physiol* **278**, C490-C499.

Vovk A, Smith WD, Paterson ND, Cunningham DA, & Paterson DH (2004). Peripheral chemoreceptor control of ventilation following sustained hypoxia in young and older adult humans. *Exp Physiol* **89**, 647-656.

Wei JY, Mendelowitz D, Anastasi N, & Rowe JW (1986). Maintenance of carotid baroreflex function in advanced age in the rat. *Am J Physiol* **250**, R1047-R1051.

Zapata P & Zuazo A (1980). Respiratory effects of dopamine-induced inhibition of chemosensory inflow. *Respir Physiol* **40**, 79-92.

**FIGURE LEGENDS**

**Figure 1.** Age-dependent variation in body weight in rats. Data represent mean±S.E.M, n=30, ***p<0.0001, Mann-Whitney U-test, compared with 3 months old rats.

**Figure 2.** Effect of ageing on basal values of respiratory frequency ($f_R$), tidal volume ($V_T$), respiratory minute volume ($V_E$), heart rate (HR) and arterial blood pressure (BP) in anesthetized and vagotomized rats (n=20). Data represent mean±S.E.M., *p<0.01; **p<0.001; ***p<0.0001, Mann-Whitney U-test vs 3 months old rats.

**Figure 3.** Effects of common carotid occlusions (CCO) on respiratory airflow (PulmFI;ml/sec) respiratory rate ($f_R$), tidal volume ($V_T$ or TV), respiratory minute volume ($V_E$) and arterial blood pressure (BP) in anestethized and vagotomized rats throughout ageing (n=10). Original recordings obtained for CCO, during 10s (A) and 15s (B) in a 3 and a 24 months old rat. (C) Dose-response curves obtained in 6 animals: (□) 3 months old, (▲) 12 months old and (■) 24 months old. 0 % effect corresponds to the values showed in Figure 2. Data represent mean±S.E.M..

**Figure 4.** Effects of i.c. cumulative bolus injections of dopamine on respiratory airflow (PulmFI;ml/sec), respiratory rate ($f_R$), tidal volume ($V_T$), respiratory minute volume ($V_E$), heart rate (HR) and arterial blood pressure (BP) in anestethized and vagotomized rats throughout ageing. Original recordings obtained for dopamine 30 nmol: (D) 3 months and (E) 24 months old rats. (A, B. C and F) dose-response curves obtained in 20 animals: (□) 3 months old, (▲) 12 months old and (■) 24 months old. 0 % effect corresponds to the values showed in Figure 2. Data represent mean±S.E.M..

**Figure 5.** Dose-response curves for the effects of cumulative i.c. bolus injections of dopamine on respiratory minute volume ($V_E$), heart rate (HR) and blood pressure (BP) in anestethized and vagotomized rats, in the absence and in the presence of i.v. infusion of domperidone. (A and D) 3 months old rats, (B) 12 months old rats and (C and E) 24 months old rats. (□) in the absence of domperidone (n=13-20); in the presence of i.v. domperidone (n=6) (▼) 0.01 mg.Kg-1.min-1; (♦) 0.1 mg.Kg-1.min-1 and (●) 0.5 mg.Kg-1.min-1. 0% increase represents absolute values determined for the 12 s that preceded the injections. Data represent mean±S.E.M., *p<0.01; **p<0.001; ***p<0.0001, Mann-Whitney U-test compared with (□).

**Figure 6.** Effect of domperidone i.v., by itself on respiratory minute volume ($V_E$), heart rate (HR) and blood pressure (BP) in anestethized and vagotomized 3 and 24 months old rats (n=6). Only one dose was tested by animal. Data represent mean±S.E.M., *p<0,01, Mann-Whitney U-test comparing 3 vs 24 months old rats.

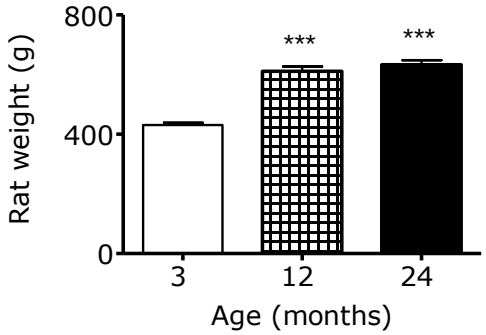
**Figure 7.** Effects of i.c. cumulative bolus injections of adenosine on respiratory airflow (PulmFI;ml/sec), respiratory rate ($f_R$), tidal volume ($V_T$), respiratory minute volume ($V_E$), heart rate (HR) and arterial blood pressure (BP) in anestethized and vagotomized rats throughout ageing. Original recordings obtained for adenosine 30 nmol: (D) 3 months and (E) 24 months old rats. (A, B,C, F and G) dose-response curves obtained in: (□) 3 months old and (■) 24 months old (n=12). 0 % effect correspond to the values showed in Figure 2. Data represent mean±S.E.M., *p<0.01; ***p<0.0001, Mann-Whitney U-test compared with (□).

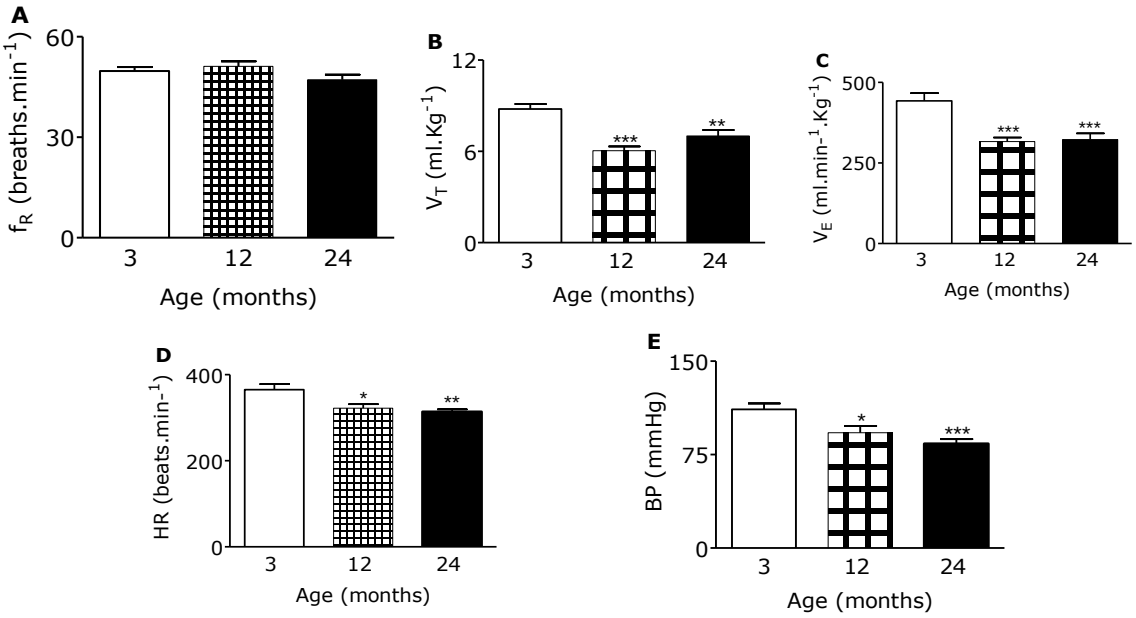
**Figure 8.** Dose-response curves for the effects of cumulative i.c. bolus injections of adenosine on respiratory minute volume (VE), heart rate (HR) and blood pressure (BP) in anestethized and vagotomized rats, in the absence and in the presence of SCH 58261 i.v.. (A, C and E) 3 months old rats; (B, D and F) 24 months old rats. (□) in the absence of SCH 58261 (n=12); in the presence of i.v. SCH 58261 (n=6) (◊) 2 ng.Kg-1.min-1 and (♦) 20 ng.Kg-1.min-1. 0% increase represents absolute values determined for the 12 s that preceded the injections of adenosine. Data represent mean±S.E.M., *p<0.01; **p<0.001; ***p<0.0001, Mann-Whitney U-test compared with □.

**Figure 9**. Effect of SCH 58261 alone (◊2 and ♦20 ng.Kg-1.min-1, i.v., 3 minutes infusion) on respiratory minute volume ($V_E$), heart rate (HR) and arterial blood pressure (BP) in anestethized and vagotomized rats. A. 3 (□) and 24 (■) months old rats (n=6). Only one dose was tested by animal. Data represent mean±S.E.M., **p<0.001, Mann-Whitney U-test compared with vehicle and between the two doses of SCH 58261. **Figure 10.** (A) Effect of ageing on cAMP levels (expressed as pmol/CB and as pmol/mg tissue) in the CB of rats (n=12-16) incubated in normoxic conditions (20% O2). (B) Comparison between the levels of cAMP in the CBs of 3 (n=14-18) and 24 (n=10-12) month old rats incubated with different oxygen concentrations. Data are mean±S.E.M.

**Figure 10.** (A) Effect of ageing on cAMP levels (expressed as pmol/CB and as pmol/mg tissue) in the CB of rats (n=12-16) incubated in normoxic conditions (20% O2). (B) Comparison between the levels of cAMP in the CBs of 3 (n=14-18) and 24 (n=10-12) month old rats incubated with different oxygen concentrations. Data are mean±S.E.M.

Monteiro, et al., Fig. 1



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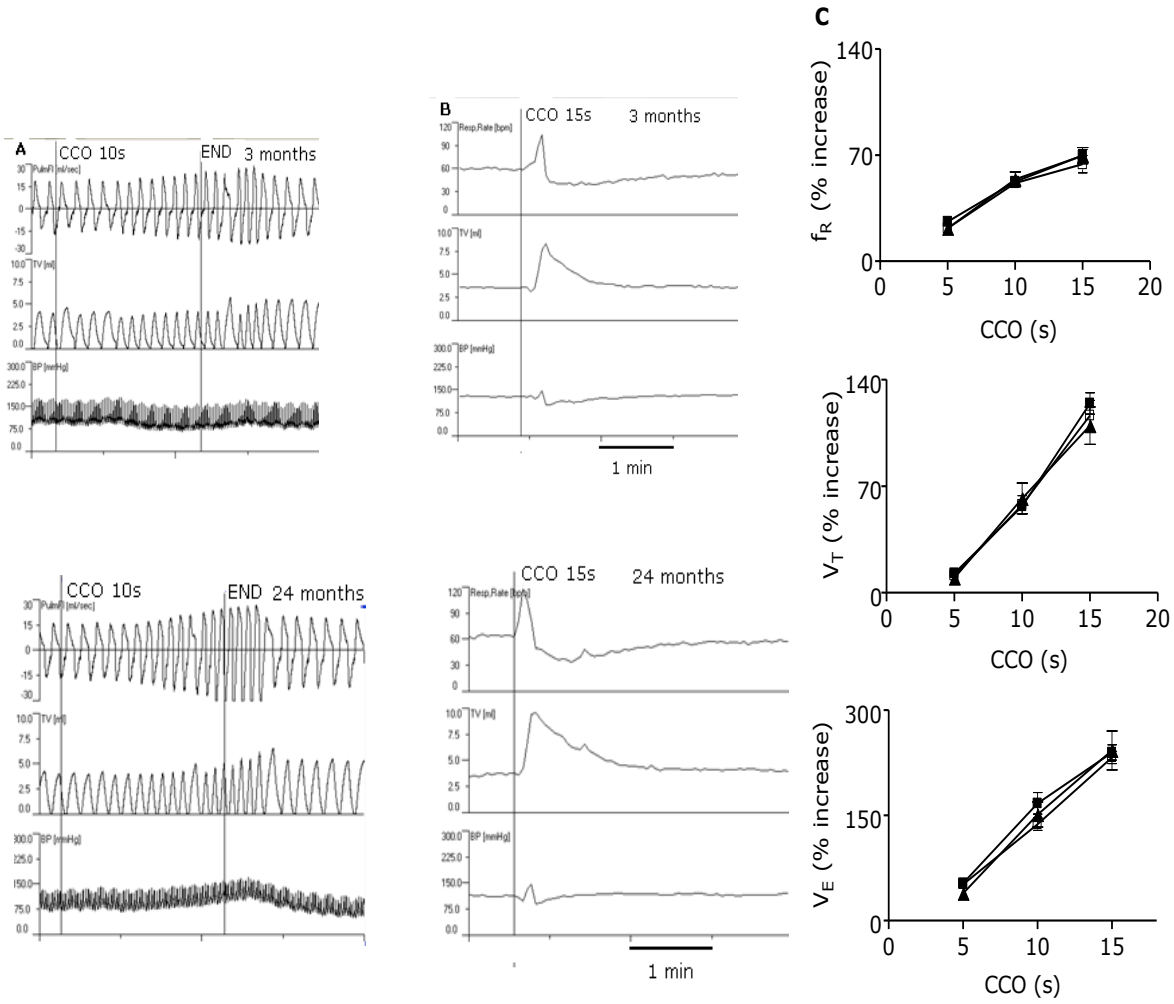


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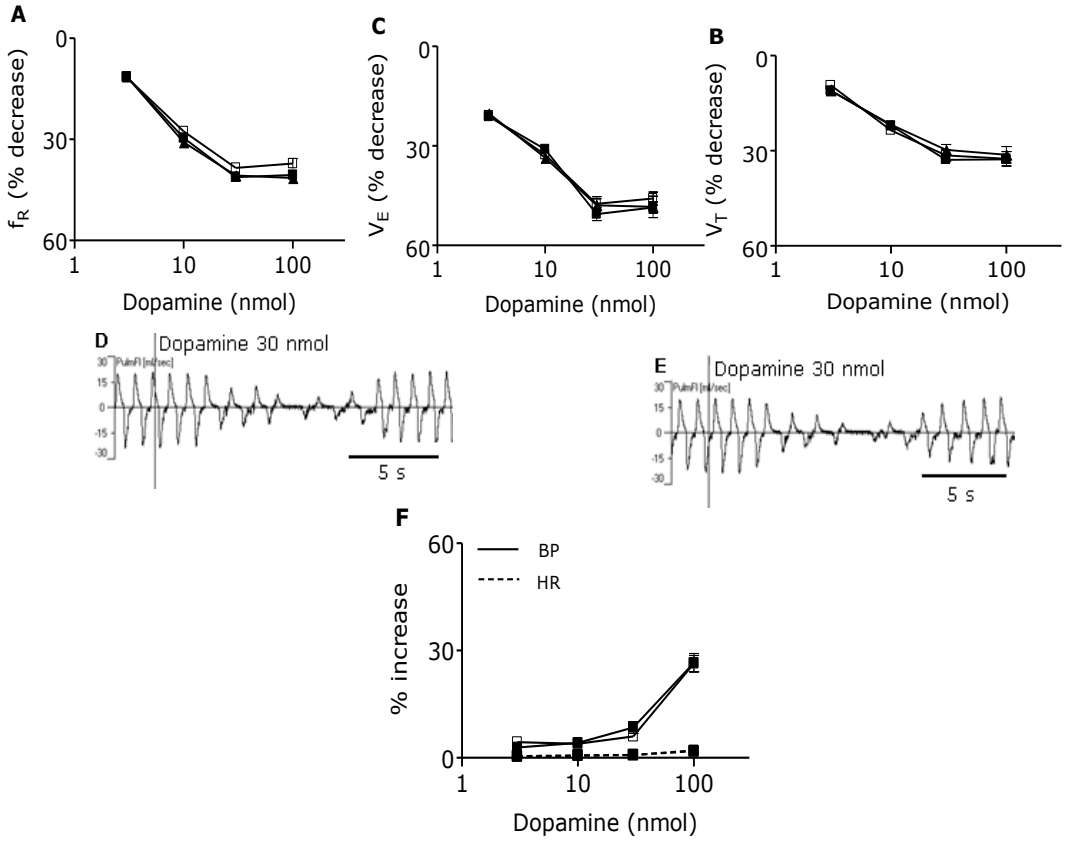


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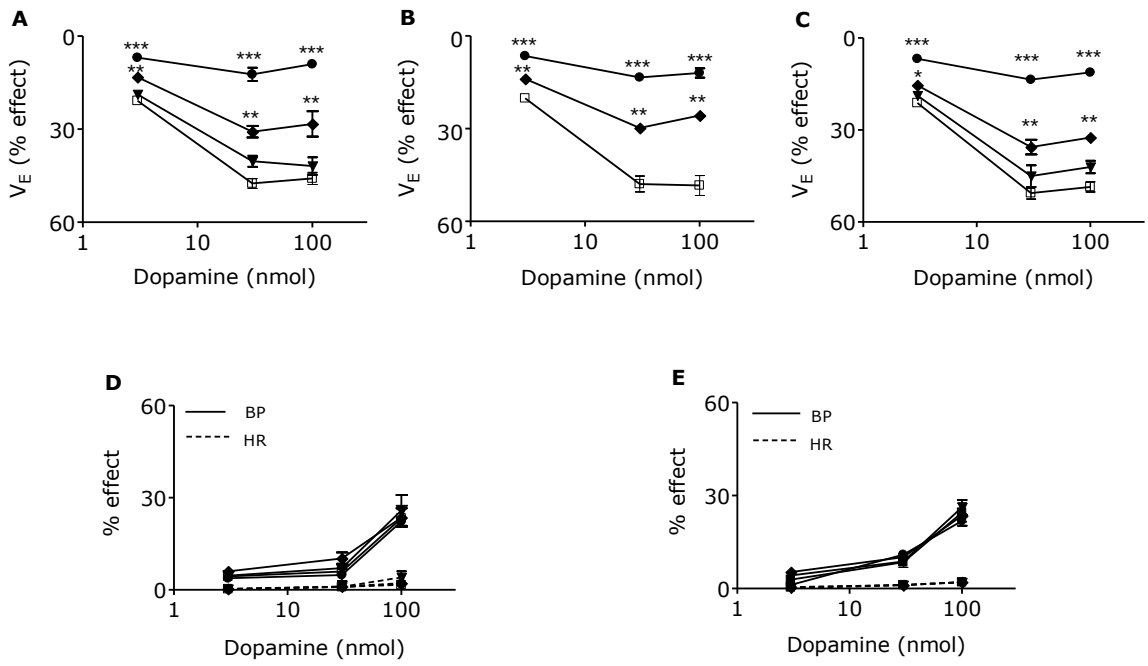


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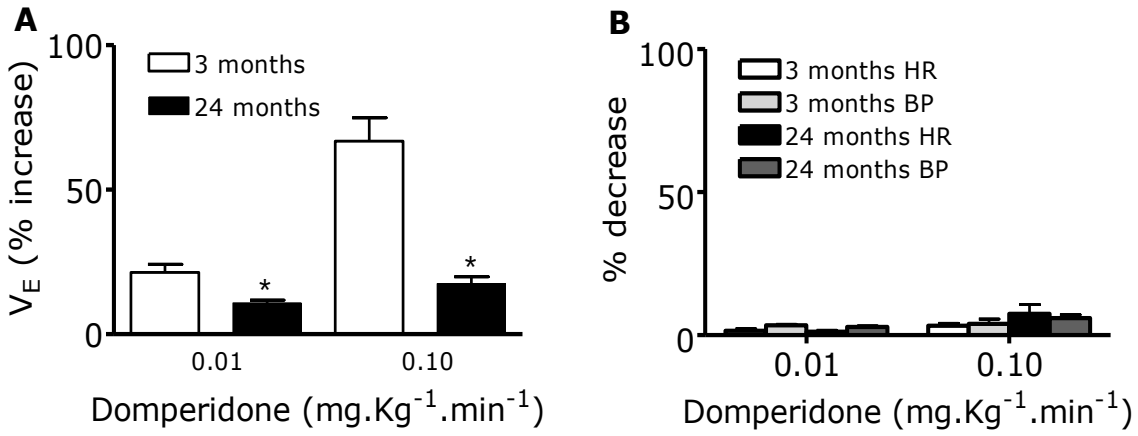


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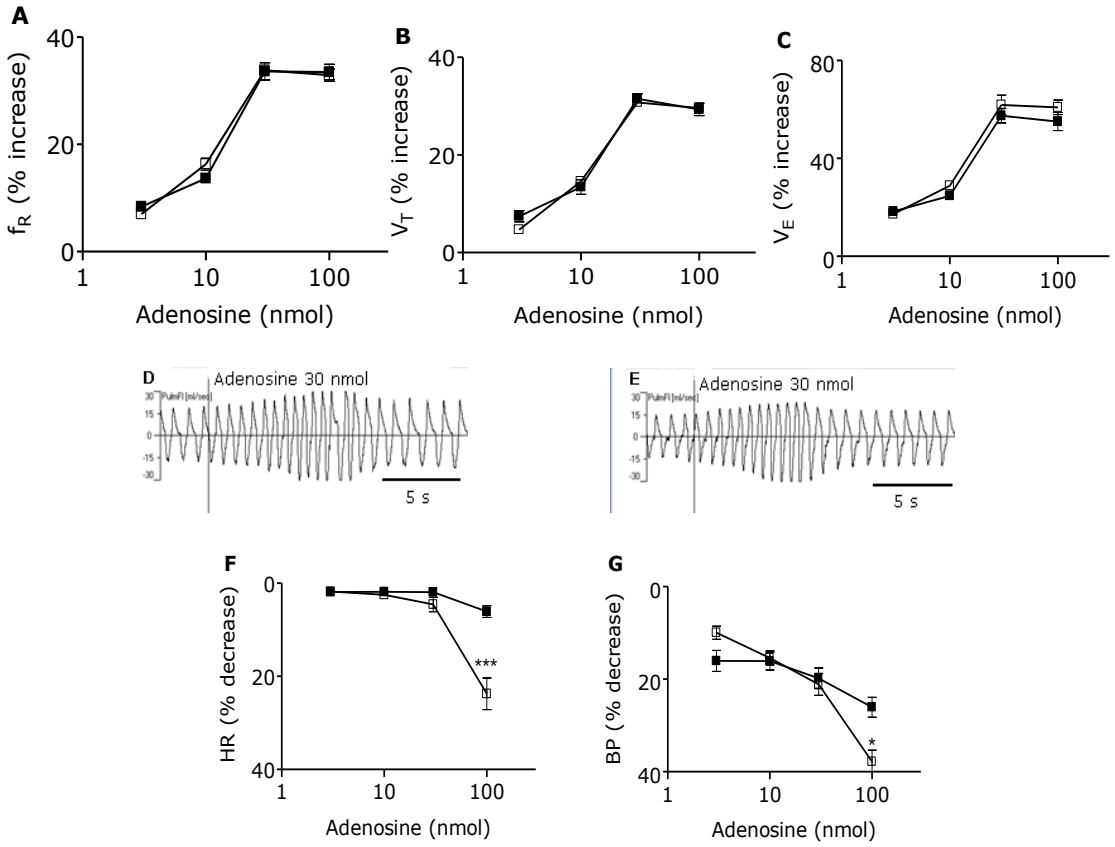


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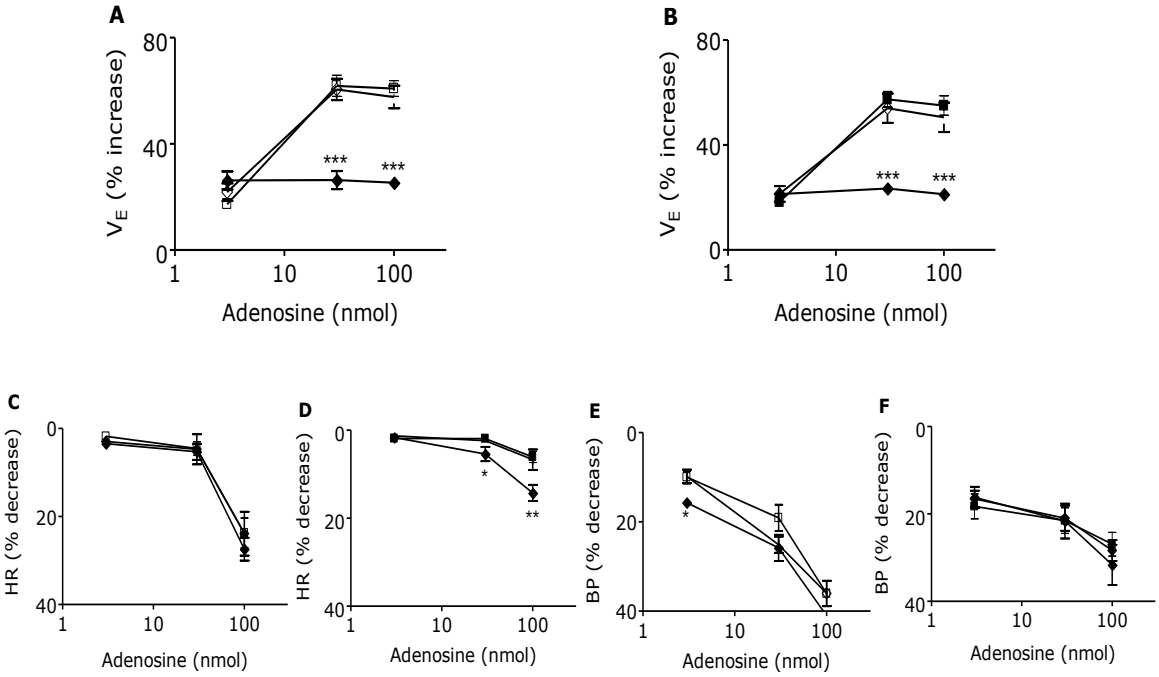


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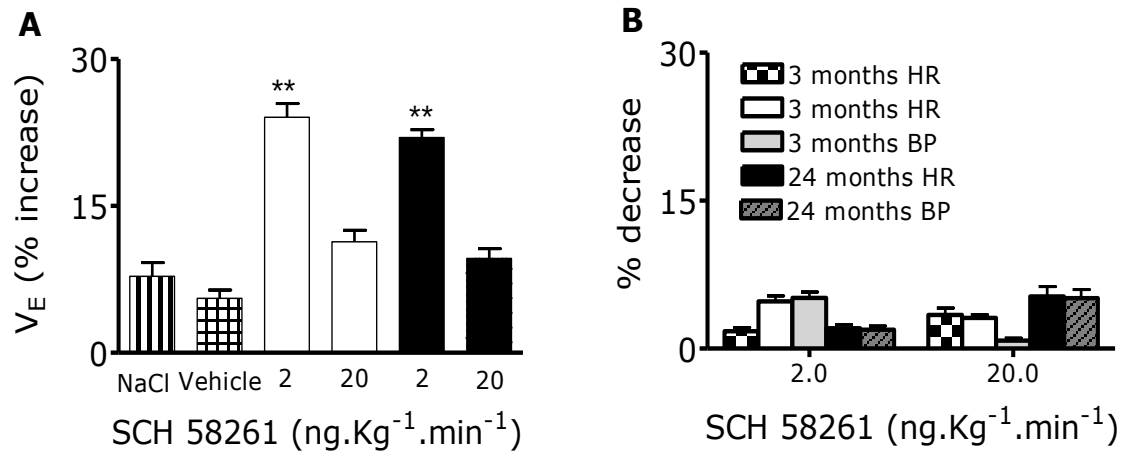


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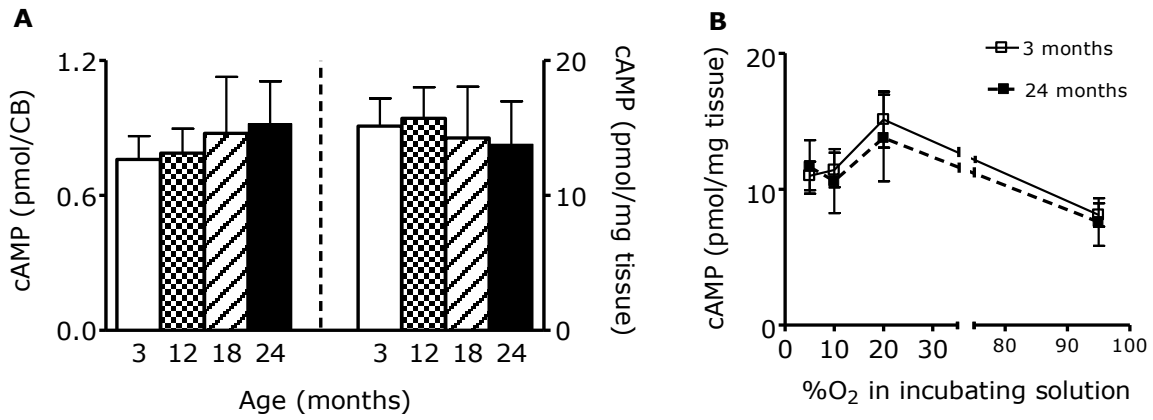


Monteiro, et al., Fig. 10

# Supplement:

## *In vivo* Experiments

**Surgery and general procedures.** The animals were anaesthetized by a single intraperitoneal injection of sodium pentobarbitone (60 $mg.Kg^{-1}$), supplemented intravenously with 10% of the initial dose as necessary, so as to make them areflexic to a nociceptive stimulus (effects of corneal reflexes and pinch to the front paw on the rise in arterial blood pressure (BP). They were placed supine, tracheostomized and breathing room air spontaneously. Bilateral midcervical vagotomy was also performed to abolish the role of afferents in the vagi innervating the lungs that had a major influence on respiratory activity (Marek et al., 2008).

Systemic BP recordings and anaesthetic supplements were performed through the introduction of steriflex catheters (Vygon 160-07 and 160-0) under a dissection microscope (Nikon SMZ-2B) in the right femoral artery and vein, respectively. All the catheters used in this experiments were filled with heparinised saline (100 $IU.ml^{-1}$; heparin sodium).

Body temperature was maintained close to 37±1°C using a heated underblanket governed by a rectal thermistor probe.

The experiments lasted approximately 8:00 and upon completion of the studies, euthanasia of the rats was achieved by iv injection of a

lethal dose of pentobarbital sodium (180 mg.Kg$^{-1}$ iv), according to standard procedures.

**Bilateral common carotid occlusions (CCO).** Both common carotid arteries were dissected approximately 1 cm below the bifurcation, and the bilateral arterial lumen was occluded by pulling simultaneously a surgical silk placed around each common carotid artery at this level, taking care to minimize stretching the carotid bifurcation. Three sequences of CCOs of 5, 10 and 15 s were performed with intervals of at least 5 min.

Control values for respiratory frequency ($f_R$), tidal volume ($V_T$), BP and heart rate (HR) correspond to the mean value measured in a period of 25 s immediately before CCO. After CCO, the values of $f_R$, $V_T$, BP and HR were taken as the maximal effects measured during the period of 25 s that followed the CCOs, and were compared with controls. The maximal effects induced by CCOs always occurred in the first 25 s that followed the end of the CCO.

For each experiment, in one rat, bilateral denervation of the CBs was performed by cutting the CSN.

**Drug administrations.** Intravenous (iv) and intracarotid (ic) drug administrations were made, respectively, into the right femoral vein (Vygon 167.10+0.5-1.0mm) and into the right common carotid artery through a catheter (Vygon 167.07+0.3-0.7mm) introduced through the external carotid artery with its tip positioned just below the

bifurcation. Infusions rate and duration were 0.5 $mL.min^{-1}$ during 3 min (Braun perfusion pump) and bolus injections made in a volume of 0.1 mL, washed in with 0.2 mL of 0.9% aqueous sodium chloride.

**Cardioventilatory parameters.** Recordings of pulmonary air flow (PulmFI;ml/sec) was obtained by a HSE-pneumotachometer PTM type, a differential pressure transducer (model DP 45-14 Validyne Engineering, NorthNorthridge, CA) and a pressure amplifier (Plugsys Housings, model 603, HSE-HA GmgH). BP was measured with a pressure transducer (model Isotec, HSE-HA GmgH) and a pressure amplifier (Plugsys Housings, model 603, HSE-HA GmgH). $V_T$ and HR were calculated by the software HSE-Harvard Pulmodyn® W, respectively from PulmFI and BP. A rectal oximetry sensor (SurgiVet V90004 Capnograph) provided a continuous monitoring of $PO_2$.

Cardioventilatory data acquisition (PulmFI, $V_T$, $f_R$, BP, HR) were obtained continuously during the experiments using the software HSE-Harvard Pulmodyn® W. Respiratory minute volume ($V_E$), defined as the product of $V_T$ and $f_R$, was further calculated. $V_T$ and $V_E$ were normalized for body weight ($mL.Kg^{-1}$ and $mL.min^{-1}.Kg^{-1}$, respectively).

**Experimental protocols with drug administrations.** Three cumulative dose-response curves for adenosine or dopamine were performed in each rat: ic bolus after 3 minute infusion of drug vehicles, 0.9% aqueous sodium chloride (in the experiments with

domperidone) or DMSO (in the experiments with SCH 58261). Two cumulative dose-response curves for adenosine or dopamine were performed in each rat: i.c. bolus after 3 minute infusion of the antagonist, domperidone (in the experiments with dopamine) or SCH 58261 (in the experiments with adenosine). Only one dose of the $D_2$ and $A_{2A}$ antagonists (domperidone or SCH 58261) was tested per animal. The intervals between drug injections or infusions were at least of 5 min. Control values for $f_R$, $V_T$, BP and HR correspond to the mean value measured in a period of 25 s immediately before drug administration. After drug ic administration, the values of $f_R$, $V_T$, BP and HR were taken as the maximal effects measured during the period of 25 s that followed the injections, and were compared with controls. The maximal effects induced by ic injections of dopamine or adenosine always occurred in the first 25 s that followed the end of the injections.

For each experiment, in one rat, bilateral denervation of the carotid bodies was performed by cutting the CSN.

### *In vitro* Experiments

**Surgery.** CBs were removed from the carotid bifurcation of anaesthetized rats with sodium pentobarbitone (60 $mg.Kg^{-1}$), tracheostomized and breathing spontaneously with the aid of a Nikon

SMZ-2B dissection scope. After removal of the CBs, the animals were killed by an intracardiac injection of a lethal dose of pentobarbital.

**Experimental protocols with cAMP.** Immediately after surgical removal, the CBs were pre-incubated for 15 min at 37 ºC in a shaker bath in medium equilibrated with 95% $O_2$ / 5% $CO_2$ to allow the recovery of the preparation. Incubation medium was a modified Krebs solution composed of: NaCl 116 mM; $NaHCO_3$ 24 mM; KCl 5 mM; $CaCl_2$ 2 mM; $MgCl_2$ 1.1 mM; HEPES 10 mM; glucose 5.5 mM; pH 7.42. After the pre-incubation period, the CBs were placed in 2 mL Eppendorf tubes containing 1 mL of fresh incubating solution of identical composition and containing, in most of the experiments, 500 μM isobutylmethylxanthine (IBMX), a phosphodiesterase inhibitor. The incubating solutions were equilibrated with 95 %$O_2$/5 %$CO_2$ (hyperoxia), 20 %$O_2$/5 %$CO_2$/75 %$N_2$ (normoxia), 10 %$O_2$/5 %$CO_2$/85 %$N_2$ (mild hypoxia) or 5 %$O_2$/5 %$CO_2$/90 %$N_2$ (moderately intense hypoxia); incubation lasted 30 min and was also carried out in the metabolic bath at 37°C. During the entire incubation period the solutions were gently bubbled with the selected gas mixture saturated with water vapor via a fine plastic tube penetrating the tubes through the caps. Extraction of cyclic nucleotides and cAMP quantification were performed as previously described by Batuca *et al.*. In brief, to extract cyclic nucleotides from the CBs, they were immersed in cold 6% (w/v) trichloroacetic acid (600μL) for 10 min, homogenised using a Potter homogeniser with a

glass and further centrifuged at 12000 g for 10 min (4 ºC). The supernatants were washed four times in 3 mL of water-saturated diethyl ether solution, the remaining aqueous phase was lyophilised, and the sealed samples were stored at -20 ºC until cAMP was assayed using an EIA commercial kit (GE Healthcare Bio-Sciences AB, Sweden) (Batuca et al., 2003) .

**Drugs**

All drugs were prepared on the day of each experiment. Doses of all drugs were calculated on the basis of salt weight. Dopamine, adenosine and domperidone were prepared in saline (0.9% NaCl). Stock solutions (5mM) of SCH 58261 was prepared in dimethylsulfoxide (DMSO) and stored at –20ºC until use. Stock solution was further diluted with saline prior to each experiment. The highest concentration of the vehicle in venous perfusion was 0.4 mM or 0.01%.